RESEARCH ARTICLE

Transcriptional profiling of *Francisella tularensis* infected peripheral blood mononuclear cells: a predictive tool for tularemia

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tularemia; microarray; gene expression; PBMCs.

Abstract

In this study, we analyzed temporal gene expression patterns in human peripheral blood mononuclear cells (PBMCs) infected with the *Francisella tularensis* live vaccine strain from 1 to 24 h utilizing a whole human Affymetrix® gene chip. We found that a considerable number of induced genes had similar expression patterns and functions as reported previously for gene expression profiling in patients with ulceroglandular tularemia. Among the six uniquely regulated genes reported for tularemia patients as being part of the alarm signal gene cluster, five, namely caspase 1, PSME2, TAP-1, GBP1, and GCH1, were induced *in vitro*. We also detected four out of the seven potential biomarkers reported in tularemia patients, namely TNFAIP6 at 4 h and STAT1, TNFSF10, and SECTM1 at 16 and 24 h. These observations underscore the value of using microarray expression profiling as an *in vitro* tool to identify potential biomarkers for human infection and disease. Our results indicate the potential involvement of several host pathways/processes in *Francisella* infection, notably those involved in calcium, zinc ion binding, PPAR signaling, and lipid metabolism, which further refines the current knowledge of *F. tularensis* infection and its effects on the human host. Ultimately, this study provides support for utilizing *in vitro* microarray gene expression profiling in human PBMCs to identify biomarkers of infection and predict *in vivo* immune responses to infectious agents.

Introduction

Francisella tularensis is a facultative intracellular Gram-negative coccobacillus that causes tularemia in humans and is considered a potential biowarfare agent due to its infectious nature. *Francisella* infection in humans is mostly due to two subspecies of *F. tularensis*, namely *tularensis* and *holoarctica*, and clinical symptoms of tularemia depend on the route of infection. Laboratory studies utilize *F. tularensis* live vaccine strain (LVS), an attenuated strain of *Francisella* derived from *holoarctica*. Ulceroglandular tularemia, acquired by a bite from an infected vector or contact with an infected animal, affects the lymphoid system the most and is characterized by a transient bacteremic phase in early infection (Ellis *et al*., 2002). Pneumonic tularemia, due to inhalation of *F. tularensis*, causes the most severe form of the disease. Typhoidal tularemia, an acute form of the disease with a high mortality rate, may induce septicemia and fever without the primary involvement of lymph nodes or lungs (Ellis *et al*., 2002).

Peripheral blood mononuclear cells (PBMCs) are powerful biological first responders to invading pathogens and, in the initial stages of infection, activate host innate immune responses (Janeway & Medzhitov, 2002). Macrophages, neutrophils, and PBMCs respond to a broad range of bacterial stimuli with common transcriptional activation programs (Boldrick *et al*., 2002; Nau *et al*., 2002). Knowledge of complex gene expression patterns in PBMCs to *F. tularensis* infection in early presymptomatic stages would be critical to the identification of potential transcriptional markers and clarification of molecular mechanisms triggering host defense. Although significant strides have been made in understanding the cell-mediated immune mechanisms and cytokine responses in *Francisella* infections, little is

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14. ABSTRACT

We studied early temporal gene expression in human peripheral blood mononuclear cells after in vitro infection with Francisella tularensisi live vaccine strain. Recently, investigators from Sweden studied ulceroglandular tularemia by gene expression profiling of blood from patients with active ongoing infection. Host responses to tularemia were described in their study of several potential genes as biomarkers for early tularemia. Most of the host genes related to tularemia were confirmed in our in vitro study, in addition to several biomarkers, indicating the value of gene erxpression profiling to identify potential biomarkers. Herein, we describe gene expression changes in the early human immune response and other cellular processes. We also report the identification of genes expressed in hitherto unknown pathways related to Francisella infection, notably those involved in zinc and lipid metabolism. Our work demonstrated the applicability of utilizing microarray gene expression as a predictor of in vivo immune responses to infectious agents.

15. SUBJECT TERMS
Franciscella tularensis, tularemia, gene expression, in vitro, early host response, microarray

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known about the transcriptional events that drive the host responses in PBMCs in early infection. To uncover the features of transcriptional events associated with *Francisella* infection in presymptomatic stages, and to compare these events with those found in active ongoing tularemia, we examined the gene expression profiles of human PBMCs in early *Francisella* infection.

Microarray technology is used as a tool to elucidate host responses *in vivo*, and this study attempts to relate *in vitro* gene expression profiling to findings from *in vivo* tularemia (Andersson *et al.*, 2006). We included uninfected controls at every time point for each individual and studied eight biological replicates. In addition to detecting gene expression patterns that were similar to the patterns found in tularemia patients, our findings indicated the potential use of gene expression profiling for biomarker selection in an active ongoing infection. Utilizing a whole human genome chip array, we identified several new host pathways in early *Francisella* infection that warrant further investigation.

Materials and methods

Subjects

After this study was approved by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) Human Use Committee, with written informed consent, eight male volunteers who were 21–44 years of age with no known history of previous exposure to *F. tularensis* were recruited from the USAMRIID Special Immunizations Program.

PBMC isolation

PBMCs were isolated from whole blood of each volunteer using a Ficoll gradient method (Sigma, St. Louis, MO). Briefly, whole blood was diluted 1 : 2 with Dulbecco's phosphate-buffered saline (PBS) (Sigma), layered on Histopaque 1077 (Sigma), and centrifuged at 500 ***g***. The resulting interphase was collected and washed twice with PBS. The final cell pellet was resuspended in RPMI-1640 with 2 mM Glutamine + 20% AB serum (Valley Biomedicals, VA). Concentration of cells for each sample ($n = 8$) was determined using a hemacytometer and adjusted accordingly.

Growth of LVS for *in vitro* infection

The LVS vaccine (NDBR101, lot number 4) was provided by USAMRIID, Fort Detrick, Frederick, MD. A live culture of *F. tularensis* LVS was grown in thioglycollate broth (BBL Becton Dickinson) supplemented with 2% Isovitalex in a 37 °C shaker incubator, and stock cultures were aliquoted in the medium and stored at − 80 °C for future use. The stock was utilized for infection at appropriate dilutions.

Infection of PBMCs with *F. tularensis* (LVS)

Infections were performed independently on PBMCs isolated from each volunteer. Cells were placed in six-well plates at a concentration of 1×10^7 cells per well and left to rest overnight at 37 °C in a 5% CO_2 incubator. LVS was used at a multiplicity of infection (MOI) of 1 : 10 (bacteria : cells) and uninfected control cells were treated with medium alone. Cells were harvested for gene expression studies at 1, 4, 8, 16, and 24 h post infection.

RNA extraction and microarray hybridization

RNA was extracted from cells at each time point using Trizol (Invitrogen) and was further purified using Qiagen RNeasy MinElute Cleanup kit (Qiagen). Quality control of RNA samples was accomplished with a Bioanalyzer 2100 (Agilent Technologies). Microarray hybridizations were performed with Affymetrix Human Genome U133 Plus 2.0 microarray chip (Affymetrix) consisting of 54 675 probe sets, encompassing 38 500 genes at the Virginia Bioinformatics Institute Core Laboratory Facility (VBI, Blacksburg, VA).

Microarray data analysis

The data were imported from 80 Affymetrix CEL files using the robust multiarray average (RMA) algorithm (Irizarry *et al.*, 2003). Expression values were $\log_2$ transformed as part of the RMA normalization. Before visualization, the transcripts were filtered to identify a subset of genes that were significantly differentially regulated over time. A twofold change cutoff was applied and two-way ANOVA was performed on the dataset (GeneSifter, VizXLabs). Next, a false discovery rate (FDR) of 5% and Tukey's HSD test (GeneSifter) were applied to the dataset with P values ≤ 0.05.

Gene ontology analysis was performed using OntoExpress (Draghici *et al.*, 2003) to create Fig. 1. Thereafter, ontology reports were generated on functional analysis with the GeneSifter software program (VizXLabs). For each function, over/under-represented genes were selected based on z-score values of the microarray data set (Doniger *et al.*, 2003) (GeneSifter, VizXLabs).

The z score is calculated by subtracting the expected number of genes in a GO term meeting the criterion from the observed number of genes, and dividing by the square root of the SD of the observed number of genes. A z score of > 2 and < 2 is considered significant for over- and under-represented genes, respectively.

$$z = \frac{(\text{observed} - \text{expected})}{\sqrt{\text{SD (observed)}}}$$

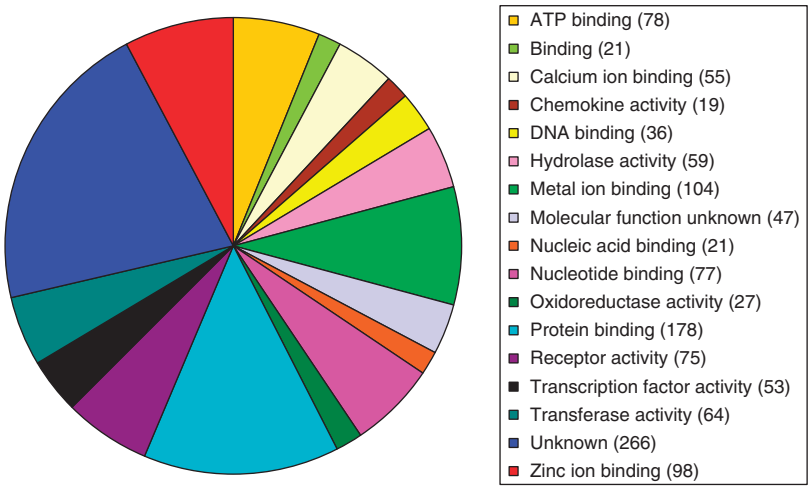

Fig. 1. All genes differentially expressed 1–24 h after infection of PBMCs with *Francisella* LVS when compared with uninfected controls ($n = 8$). Genes were identified based on OntoExpress. Numbers in parenthesis indicate statistically significant transcripts (Affymetrix probe set IDs) associated with each function.

or

$$z = \frac{\left(r - n\frac{R}{N}\right)}{n\left(\frac{R}{N}\right)\left(1 - \left(\frac{R}{N}\right)\left(1 - \frac{n-1}{N-1}\right)\right)}$$

where R, total number of genes meeting selection criteria; N, total number of genes measured; r, number of genes meeting selection criteria with the specified GO term; n, total number of genes measured with the specific GO term (GeneSifter, VizXLabs) (Doniger *et al.*, 2003).

Results

Early host response to *F. tularensis*

To identify host genes that were differentially expressed early in response to *F. tularensis*, PBMCs from each study volunteer were exposed to *Francisella tularensis* and also to a time-matched control treatment of media alone. The number of intracellular bacteria and the levels of selected secreted cytokines and chemokines have been described for the infected PBMCs in an earlier manuscript (Paranavitana *et al.*, unpublished data). At 1 h post infection using an MOI of 1 : 10 (bacteria : cells), we did not detect significantly expressed genes in infected cells. By 4 h, 24 genes (Affymetrix probe IDs) were significantly upregulated in PBMCs exposed to *Francisella* (Table 1). At this time, cells responded to the invading pathogen with increased transcription of IL1β, MIP1α, MIP1β, IL8, tumor necrosis factor (TNF), and TNFAIP6. Interestingly, IL1β protein was detected only at 8 h in the supernatants of these cells (Paranavitana *et al.*, unpublished data). IL1Ra was upregulated as early as 4 h, and we reported its secretion starting at 8 h (Paranavitana *et al.*, unpublished data). ILRa inhibits the activities of IL1α and IL1β and modulates IL1-related immune and inflammatory responses. Early innate immune responses were activated as indicated by upregulation of TLR2 and CD80 transcripts at 4 h post infection. These results are in agreement with previous work that indicated LVS-infected dendritic cells induced CD80, CD40, and inflammatory cytokines via TLR2 signaling (Katz *et al.*,

Table 1. Earliest genes (4 h) induced in PBMCs after infection with *Francisella tularensis* LVS

Accession number	Gene ID	Fold change*	SEM†	*P* value‡
NM_001995	ACSL1	1.15	0.06	0.0289
NM_001674	ATF3	1.18	0.07	0.0228
NM_002983	CCL3 (MIP1α)	1.30	0.10	0.0011
NM_002984	CCL4 (MIP1β)	1.22	0.06	0.0006
AK024901	cDNA	1.14	0.04	0.0254
BC042665	CD80	1.17	0.07	0.0028
NM_005306	FFAR2 (GPR43)	1.36	0.12	0.0045
AL575512	IBRDC2	1.20	0.10	0.0293
NM_000201	ICAM1	1.18	0.06	0.0135
M15330	IL1B	1.40	0.17	0.0264
NM_000584	IL8	1.17	0.07	0.0009
AW083357	IL1Ra	1.13	0.05	0.0167
AI246590	IRAK2	1.19	0.08	0.0445
AF153820	KCNJ2	1.28	0.11	0.0499
BC005297	KMO	1.16	0.05	0.0073
AL389942	LOC285628	1.21	0.07	0.0058
AK001017	NBN	1.20	0.07	0.0298
BE646573	NFKBIZ	1.14	0.04	0.0025
AI984040	PLSCR1	1.27	0.09	0.0359
AL050388	SOD2	1.43	0.17	0.0152
NM_000594	TNF	1.32	0.08	0.0001
AW188198	TNFAIP6	1.36	0.14	0.0397
NM_003264	TLR2	1.12	0.05	0.0373
AB051513	ZC3H12C	1.29	0.10	0.0004

*Fold change represents the mean $\log_2$ ratios of *Francisella tularensis* infected/uninfected PBMC transcripts which were statistically significant in this study (two-way ANOVA, twofold cutoff; 5% FDR; $P \leq 0.05$ Tukey's HSD test) ($n = 8$).

†SEM, SE of mean.

‡*P* value determined by Tukey's HSD test (GeneSifter).

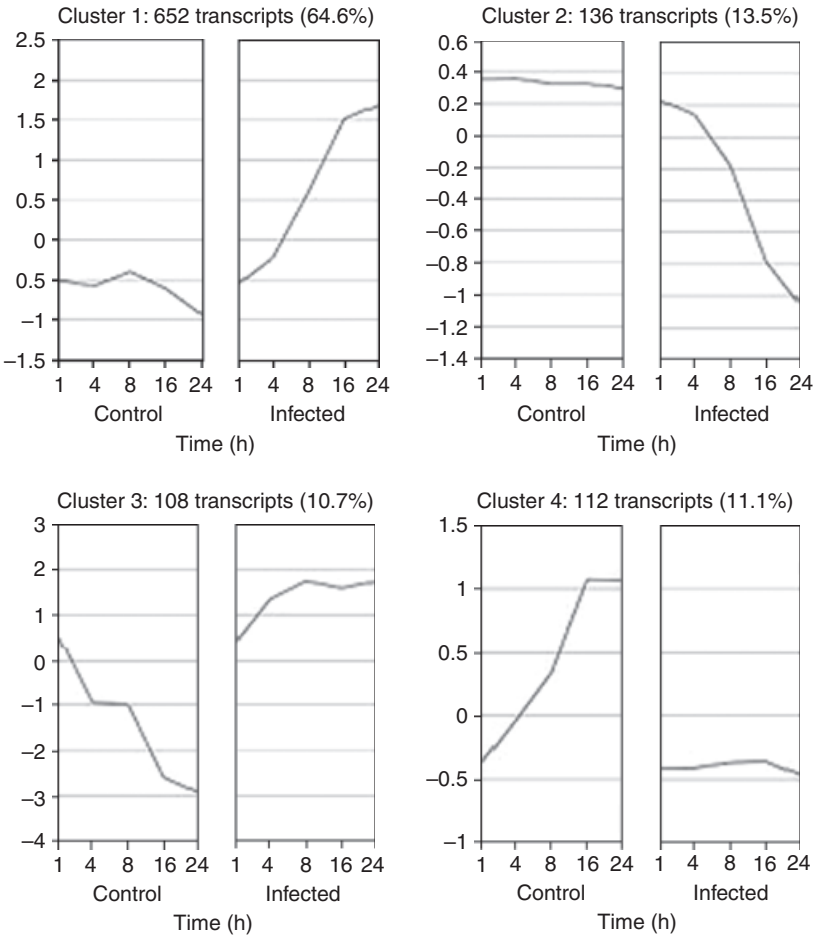

Fig. 2. Cluster analysis of gene expression in both infected and uninfected samples. K-means medoids clustering was performed for 1008 genes (Affymetrix probe set IDs) selected from two-way ANOVA with a twofold cut off and 5% FDR; $P \leq 0.05$ Tukey's HSD test) ($n = 8$). *Y*-axis = Log_2 intensity.

2006); the involvement of TLR2 in *Francisella* infection *in vivo* has been documented (Li *et al.*, 2006). We saw the induction of FFAR2 (GPR43) as early as 4 h post infection. GPR43, a highly selective fatty acid receptor of leukocytes, especially in peripheral polymorph nuclear cells (PMNs), may also play a role in differentiation and/or activation of leukocytes (Le Poul *et al.*, 2003) and in recruitment of PMNs toward sites of bacterial infection (Hong *et al.*, 2005). Phospholipid scramblase (PLSCR1) was another gene induced early (4 h). PLSCR1 is a novel interferon-stimulated gene (ISG) (Zhou *et al.*, 2002) that promotes rapid transbilayer movement of phospholipids in response to Ca^{2+} and is induced by IFNα, β, and γ (Der *et al.*, 1998). The presence of these immediate early transcripts is indicative of host cell membrane-signaling processes related to immune responses.

Temporal changes in gene expression induced by *F. tularensis*

Significant transcripts were selected by two-way ANOVA that had a twofold increase or decrease in gene expression as compared with uninfected controls. To the raw *P* values, 5% FDR (Benjamini & Hochberg, 1995) and Tukey's HSD tests were applied to obtain the final list of significant genes. A total of 1008 genes (Affymetrix probe IDs) were differentially expressed between 1 and 24 h post infection. Of these, 760 (75.3%) were upregulated, 136 (13.5%) downregulated, and 112 (11.1%) remained unchanged. Gene ontology analysis (Doniger *et al.*, 2003; Draghici *et al.*, 2003) identified binding (DNA, metal ion, nucleic acid, nucleotide, calcium, zinc, protein, ATP), catalytic (transferase, oxidoreductase, hydrolase activities), and receptor activities, among others (Fig. 1).

Genes with similar expression profiles were further grouped together by K-means medoids clustering utilizing GeneSifter software, resulting in four clusters (mean silhouette width 0.674) (Fig. 2). The first cluster of 652 genes (Affy probe IDs) from infected cells included the majority of upregulated transcripts over a period of 24 h. These genes displayed a steep increase in expression between 4 and 16 h, which increased further by 24 h (Fig. 2). Immune response and apoptosis were well represented (Table 2). Early recruitment of inflammatory cells and production of proinflammatory cytokines (IL18, IL12p40, IFNγ) are crucial for innate host defense against systemic LVS infection (Leiby *et al.*, 1992; Sjöstedt *et al.*, 1994; Elkins *et al.*, 2003). These

Table 2. Gene ontology classification and expression ratios over time in PBMCs infected with *Francisella tularensis*

		Fold change over time*				
		Time (h)				
Accession number	Gene ID	1 ± SEM	4 ± SEM	8 ± SEM	16 ± SEM	24 ± SEM
Immune response (BP)	*Cluster 1*					
NM_001250	CD40	1.02 ± 0.05	1.15 ± 0.05	**1.20 ± 0.06**	**1.49 ± 0.06**	**1.55 ± 0.04**
BC042665	CD80	1.01 ± 0.07	**1.17 ± 0.07**	**1.32 ± 0.10**	**1.59 ± 0.11**	**1.78 ± 0.10**
NM_019604	CRTAM	1.00 ± 0.06	0.99 ± 0.05	0.99 ± 0.05	1.12 ± 0.07	**1.25 ± 0.05**
NM_005755	EBI3	1.00 ± 0.11	1.08 ± 0.09	1.22 ± 0.09	**1.57 ± 0.10**	**1.69 ± 0.09**
NM_006705	GADD45G	1.00 ± 0.02	0.98 ± 0.01	1.07 ± 0.03	**1.18 ± 0.04**	**1.28 ± 0.05**
NM_001548	IFIT1	0.99 ± 0.09	1.12 ± 0.10	1.25 ± 0.11	1.26 ± 0.10	**1.42 ± 0.10**
AI075407	IFIT3	0.10 ± 0.07	1.20 ± 0.09	1.20 ± 0.08	**1.31 ± 0.08**	**1.50 ± 0.09**
M29383	IFNg	0.95 ± 0.04	1.08 ± 0.05	**1.35 ± 0.10**	**2.3 ± 0.11**	**2.94 ± 0.13**
K03122	IL2Rá	1.01 ± 0.05	1.01 ± 0.05	1.06 ± 0.03	1.18 ± 0.04	1.42 ± 0.05
NM_002187	IL12p40	1.00 ± 0.04	1.23 ± 0.09	**1.86 ± 0.22**	**2.16 ± 0.19**	**2.32 ± 0.21**
NM_000585	IL15	0.98 ± 0.06	1.04 ± 0.07	1.17 ± 0.08	**1.40 ± 0.07**	**1.53 ± 0.06**
NM_001562	IL18	0.98 ± 0.04	1.13 ± 0.07	**1.29 ± 0.09**	**1.36 ± 0.10**	**1.28 ± 0.08**
NM_002286	LAG3	0.99 ± 0.03	1.02 ± 0.02	1.09 ± 0.04	**1.31 ± 0.07**	**1.42 ± 0.08**
AA897516	PTGER4	0.99 ± 0.02	1.04 ± 0.02	**1.09 ± 0.02**	**1.15 ± 0.03**	**1.19 ± 0.03**
Apoptosis (BP)						
BC006121	AIFM2	1.02 ± 0.05	1.05 ± 0.03	1.10 ± 0.04	**1.27 ± 0.06**	**1.35 ± 0.06**
AI719655	CASP1	1.00 ± 0.03	1.04 ± 0.04	1.08 ± 0.04	**1.15 ± 0.03**	**1.19 ± 0.03**
AL050391	CASP4	0.99 ± 0.04	1.05 ± 0.04	1.08 ± 0.04	**1.23 ± 0.04**	**1.22 ± 0.04**
NM_004347	CASP5	0.98 ± 0.07	1.01 ± 0.06	1.09 ± 0.04	**1.27 ± 0.05**	**1.36 ± 0.04**
Z70519	FAS	0.99 ± 0.04	1.06 ± 0.05	**1.19 ± 0.05**	**1.52 ± 0.08**	**1.62 ± 0.06**
J03189	GZMB	1.00 ± 0.04	0.99 ± 0.04	1.00 ± 0.04	1.13 ± 0.06	**1.34 ± 0.08**
U57059	TNFSF10	0.99 ± 0.09	1.11 ± 0.11	1.22 ± 0.11	**1.36 ± 0.10**	**1.50 ± 0.05**
NM_001561	TNFRSF9	1.00 ± 0.04	1.10 ± 0.05	1.12 ± 0.04	**1.41 ± 0.05**	**1.61 ± 0.07**
Receptor activity (MF)	*Cluster 2*					
W95035	CD36	1.03 ± 0.12	0.89 ± 0.11	0.76 ± 0.07	**0.57 ± 0.03**	**0.57 ± 0.03**
NM_001736	C5AR1	1.00 ± 0.02	0.98 ± 0.03	0.96 ± 0.04	0.88 ± 0.04	**0.85 ± 0.04**
NM_005211	CSF1R	1.00 ± 0.02	0.97 ± 0.02	0.93 ± 0.03	**0.89 ± 0.03**	**0.85 ± 0.02**
AI458949	IFNGR1	1.00 ± 0.03	0.97 ± 0.05	0.91 ± 0.03	**0.83 ± 0.03**	**0.79 ± 0.03**
Transferase activity (MF)						
NM_022766	CERK	0.99 ± 0.01	0.98 ± 0.02	**0.97 ± 0.02**	**0.90 ± 0.02**	**0.90 ± 0.02**
NM_005114	HS3ST1	1.00 ± 0.02	1.00 ± 0.03	0.99 ± 0.03	0.92 ± 0.05	**0.76 ± 0.07**
NM_006343	MERTK	0.98 ± 0.03	0.97 ± 0.03	**0.94 ± 0.04**	**0.82 ± 0.06**	**0.72 ± 0.06**
Hydrolase activity (MF)						
NM_024837	ATP8B4	0.99 ± 0.06	0.92 ± 0.06	0.93 ± 0.05	**0.75 ± 0.04**	**0.71 ± 0.04**
NM_022355	DPEP2	1.01 ± 0.03	0.96 ± 0.03	0.91 ± 0.04	**0.78 ± 0.03**	**0.79 ± 0.04**
NM_014214	IMPA2	1.00 ± 0.06	0.91 ± 0.04	**0.83 ± 0.04**	**0.74 ± 0.04**	**0.72 ± 0.04**
NM_005615	RNASE6	0.99 ± 0.09	0.93 ± 0.07	0.83 ± 0.05	**0.67 ± 0.05**	**0.61 ± 0.05**
Mg ion binding (MF)						
NM_024837	ATP8B4	0.99 ± 0.06	0.92 ± 0.06	0.93 ± 0.05	**0.75 ± 0.04**	**0.71 ± 0.04**
NM_014214	IMPA2	1.00 ± 0.06	0.91 ± 0.04	**0.83 ± 0.04**	**0.74 ± 0.04**	**0.72 ± 0.04**
NM_021209	NLRC4	1.01 ± 0.03	0.93 ± 0.02	0.91 ± 0.02	**0.85 ± 0.02**	**0.81 ± 0.03**
Nucleic acid binding (MF)						
NM_000332	ATXN1	0.99 ± 0.01	0.98 ± 0.01	0.98 ± 0.02	0.90 ± 0.02	**0.89 ± 0.02**
AB051493	KIAA1706	1.00 ± 0.03	0.96 ± 0.02	0.92 ± 0.03	**0.88 ± 0.02**	**0.88 ± 0.02**
AB011110	POLR2J2	0.99 ± 0.04	0.99 ± 0.04	0.94 ± 0.05	**0.82 ± 0.04**	**0.82 ± 0.03**
NM_002957	RXRA	0.99 ± 0.01	0.96 ± 0.01	**0.94 ± 0.01**	**0.87 ± 0.02**	**0.84 ± 0.02**
AI139569	SWAP70	0.98 ± 0.01	1.01 ± 0.01	0.98 ± 0.01	**0.91 ± 0.02**	**0.89 ± 0.03**
Chemotaxis (BP)	*Cluster 3*					
S69738	CCL2	1.00 ± 0.06	1.05 ± 0.02	1.06 ± 0.02	**1.17 ± 0.04**	**1.21 ± 0.06**
NM_002983	CCL3	0.99 ± 0.06	**1.30 ± 0.10**	**1.34 ± 0.09**	**1.37 ± 0.07**	**1.42 ± 0.09**
NM_002984	CCL4	0.99 ± 0.04	**1.22 ± 0.06**	**1.30 ± 0.06**	**1.35 ± 0.05**	**1.37 ± 0.06**
NM_006273	CCL7	0.99 ± 0.04	1.12 ± 0.06	1.23 ± 0.09	**1.84 ± 0.33**	**2.16 ± 0.46**

Table 2. Continued.

		Fold change over time*				
		Time (h)				
Accession number	Gene ID	1 ± SEM	4 ± SEM	8 ± SEM	16 ± SEM	24 ± SEM
NM_004591	CCL20	0.94 ± 0.12	1.49 ± 0.27	**1.60 ± 0.26**	**1.76 ± 0.26**	**1.84 ± 0.30**
NM_001511	CXCL1	0.96 ± 0.07	1.22 ± 0.11	1.28 ± 0.11	**1.47 ± 0.18**	**1.54 ± 0.22**
NM_000576	IL1B	0.96 ± 0.09	1.37 ± 0.16	1.44 ± 0.15	**1.56 ± 0.15**	**1.58 ± 0.16**
NM_000584	IL8	0.98 ± 0.04	1.17 ± 0.07	1.19 ± 0.06	**1.37 ± 0.10**	**1.42 ± 0.13**
Signal transduction (BP)						
AF237762	GPR84	0.97 ± 0.10	1.25 ± 0.13	1.31 ± 0.12	**1.55 ± 0.13**	**1.62 ± 0.16**
AW083357	IL1Ra	1.00 ± 0.04	**1.13 ± 0.05**	**1.29 ± 0.08**	**1.27 ± 0.07**	**1.30 ± 0.05**
AI246590	IRAK2	0.99 ± 0.06	**1.19 ± 0.08**	**1.13 ± 0.07**	**1.23 ± 0.05**	**1.25 ± 0.05**
NM_000201	ICAM1	1.00 ± 0.06	**1.18 ± 0.06**	**1.21 ± 0.05**	**1.39 ± 0.06**	**1.40 ± 0.05**
NM_003264	TLR2	0.99 ± 0.03	**1.12 ± 0.05**	**1.16 ± 0.04**	**1.15 ± 0.04**	**1.15 ± 0.04**

*Fold change represents $\log_2$ mean intensities (± $\log_2$ SEM) of *Francisella tularensis* infected/uninfected PBMC transcripts ($n = 8$).
Values in bold are significant ($P \leq 0.05$) (Tukey's HSD test; GeneSifter, VizXlabs).
This is a list of selected genes.
BP, biological process; MF, molecular function.

proinflammatory cytokines with increased expression patterns were represented in cluster 1 (Table 2). Regulation of lymphocyte proliferation was indicated by upregulation of IL12p40, IL18, EBI3, IL15, and IL2Rα genes and regulation of T-cell activation was indicated by expression of LAG3, GADD45G, and CRTAM (Table 2). The transcript for prostaglandin receptor EP4 (PTGER4) increased significantly ($P \leq 0.05$) starting at 8 h. Although not statistically significant ($P = 0.065$), the expression of prostaglandin–endoperoxide synthase 2 (PTGS2) (COX-2) was slightly increased at 24 h, indicating the possible induction of PGE2 (data not shown). PGE2 secretion by *Francisella* infected macrophages has been associated with the regulation of T-cell proliferation (Woolard *et al.*, 2007).

In addition, cytokine–chemokine receptor interactions were highly represented in cluster 1, together with binding activities (Ca^{2+} and Zn^{2+}) and catalytic activities (oxidoreductase and hydrolase) (data not shown). Both apoptosis and programmed cell death related genes were upregulated and highly enriched, showing an increase in expression mostly between 16 and 24 h (TNFSF10, TNFRSF9, CASP 1, CASP 4, CASP 5, AIFM2) (Table 2). These results support previous findings in murine macrophages describing the induction of apoptosis following *Francisella* infection (Lai *et al.*, 2001; Shao *et al.*, 2005).

In the second cluster of 136 genes (Affy probe IDs) (cluster 2, Fig. 2), overall gene expression decreased over a period of 24 h post infection and the expression pattern remained almost unaffected in uninfected control cells. These genes had a slight decline in expression between 1 and 4 h, followed by a more dramatic drop thereafter (Fig. 2). *Francisella* infection caused the downregulation of transcripts related to receptor, transferase, and hydrolase activities (Table 2). The decreased expression of binding activity post infection was especially noted in a number of genes involved in nucleic acid and Mg^{2+} binding (Table 2), Ca^{2+} binding (Table 3), and ATP binding (data not shown).

The third cluster represented 108 very early host genes induced by *Francisella* infection (Fig. 2). In this group, gene transcription increased sharply between 1 and 8 h post infection, and its induction was sustained up to 24 h. In contrast, gene expression in uninfected cells showed a gradual decrease. These earliest biological processes affected by infection included chemotaxis and signal transduction (Table 2). Genes associated with apoptosis, such as TNFAIP6 and NFKBIA, were also upregulated early. Of note, starting early, metal ion-binding genes (data not shown) including zinc ion-binding genes were induced (Table 3).

The fourth cluster (112 genes) (Affy probe IDs) consisted of transcripts whose expression was increased only in the control group. Gene expression levels were relatively unchanged during the 24 h infection. The increased transcripts in uninfected cells were associated with regulation of physiological processes, development, metabolism, binding, and catalytic activities (data not shown).

In vitro corroboration of differentially expressed genes reported previously for human tularemia

TAP1 was induced in human tularemia patients. *In vitro*, its induction started at 8 h and was significant at 16 h (Table 4). The expression of GZMB was detected at 24 h and also expressed in tularemia patients (Andersson *et al.*, 2006). We

Table 3. Transcriptional profiles associated with novel pathways/processes/functions following exposure of PBMCs to *Francisella tularensis*

		Fold change*				
		Time (h)				
Accession number	Gene ID	1 ± SEM†	4 ± SEM	8 ± SEM	16 ± SEM	24 ± SEM
Tryptophan metabolism (KP)						
NM_001752	CAT	1.00 ± 0.03	0.97 ± 0.03	**0.93 ± 0.02**	**0.84 ± 0.02**	**0.83 ± 0.02**
NM_000414	HSD17B4	1.02 ± 0.12	**1.20 ± 0.10**	**1.34 ± 0.09**	**1.41 ± 0.08**	**1.44 ± 0.11**
AL575512	IBRDC2	1.02 ± 0.12	1.20 ± 0.10	**1.34 ± 0.09**	**1.41 ± 0.08**	**1.44 ± 011**
M34455	INDO	0.99 ± 0.04	1.10 ± 0.07	**1.23 ± 0.08**	**1.69 ± 0.10**	**1.95 ± 0.07**
NM_003937	KYNU	0.99 ± 0.03	1.09 ± 0.03	**1.14 ± 0.03**	**1.20 ± 0.03**	**1.17 ± 0.05**
M61715	WARS	1.00 ± 0.03	0.96 ± 0.02	0.92 ± 0.02	**0.85 ± 0.02**	**0.85 ± 0.02**
PPAR-signaling pathway (KP)						
NM_001995	ACSL1	0.98 ± 0.06	**1.15 ± 0.06**	**1.25 ± 0.06**	**1.27 ± 0.04**	**1.26 ± 0.03**
AW173691	ACSL5	0.99 ± 0.05	1.04 ± 0.04	1.10 ± 0.04	**1.16 ± 0.02**	**1.16 ± 0.03**
W95035	CD36	1.03 ± 0.12	0.89 ± 0.11	0.76 ± 0.07	**0.57 ± 0.03**	**0.57 ± 0.03**
NM_001442	FABP4	1.03 ± 0.09	0.99 ± 0.09	0.93 ± 0.08	0.76 ± 0.07	**0.64 ± 0.09**
NM_001444	FABP5	1.01 ± 0.05	0.97 ± 0.05	0.88 ± 0.04	**0.74 ± 0.03**	**0.75 ± 0.03**
NM_005693	NR1H3	1.02 ± 0.05	1.05 ± 0.05	1.15 ± 0.05	**1.23 ± 0.06**	**1.19 ± 0.06**
NM_002957	RXRA	0.99 ± 0.01	0.96 ± 0.01	**0.94 ± 0.01**	**0.87 ± 0.02**	**0.84 ± 0.02**
Lipid metabolic process (BP)						
NM_001995	ACSL1	0.98 ± 0.06	**1.15 ± 0.06**	**1.25 ± 0.06**	**1.27 ± 0.04**	**1.26 ± 0.03**
AW173691	ACSL5	0.99 ± 0.05	1.04 ± 0.04	1.10 ± 0.04	**1.16 ± 0.02**	**1.16 ± 0.03**
AF323540	APOL1	1.01 ± 0.04	1.02 ± 0.04	1.09 ± 0.05	**1.28 ± 0.03**	**1.36 ± 0.03**
BC004395	APOL2	1.02 ± 0.02	1.02 ± 0.02	1.06 ± 0.03	**1.21 ± 0.03**	**1.23 ± 0.03**
NM_014349	APOL3	1.01 ± 0.04	1.08 ± 0.05	**1.14 ± 0.06**	**1.30 ± 0.03**	**1.37 ± 0.02**
AF305226	APOL4	1.01 ± 0.06	1.01 ± 0.05	1.02 ± 0.04	**1.39 ± 0.08**	**1.52 ± 0.07**
BM980001	APOL6	1.00 ± 0.04	1.03 ± 0.04	1.10 ± 0.05	**1.25 ± 0.04**	**1.31 ± 0.04**
W95035	CD36	1.03 ± 0.12	0.89 ± 0.11	0.76 ± 0.07	**0.57 ± 0.03**	**0.57 ± 0.03**
NM_001442	FABP4	1.03 ± 0.09	0.99 ± 0.09	0.93 ± 0.08	0.76 ± 0.07	**0.64 ± 0.09**
BC021229	SELI	0.98 ± 0.03	1.05 ± 0.03	**1.13 ± 0.04**	**1.21 ± 0.05**	**1.30 ± 0.06**
Calcium ion binding (MF)						
AA769818	CACNA1A	0.98 ± 0.05	1.04 ± 0.07	1.25 ± 0.11	**1.38 ± 0.09**	**1.38 ± 0.07**
AI093579	ITGAV	0.98 ± 0.03	1.01 ± 0.03	1.00 ± 0.03	0.91 ± 0.05	**0.85 ± 0.05**
NM_002664	PLEK	1.00 ± 0.02	1.06 ± 0.02	**1.08 ± 0.02**	**1.16 ± 0.02**	**1.21 ± 0.02**
NM_002961	S100A4	1.02 ± 0.04	0.98 ± 0.03	0.94 ± 0.02	**0.83 ± 0.03**	**0.80 ± 0.02**
BI825302	TMEM37	1.01 ± 0.02	0.98 ± 0.01	0.91 ± 0.02	**0.67 ± 0.04**	**0.67 ± 0.03**
NM_017901	TPCN1	0.99 ± 0.02	0.95 ± 0.03	0.90 ± 0.03	**0.82 ± 0.02**	**0.78 ± 0.03**
NM_004385	VCAN	0.99 ± 0.03	0.98 ± 0.05	0.91 ± 0.06	0.84 ± 0.08	**0.71 ± 0.06**
Zinc ion binding (MF)						
M10943	MT1F	0.99 ± 0.05	1.02 ± 0.06	1.04 ± 0.06	**1.24 ± 0.07**	**1.33 ± 0.08**
NM_005950	MT1G	0.98 ± 0.07	1.03 ± 0.07	1.05 ± 0.07	**1.30 ± 0.10**	**1.39 ± 0.11**
NM_005951	MT1H	0.98 ± 0.06	1.03 ± 0.06	1.05 ± 0.06	**1.29 ± 0.09**	**1.40 ± 0.11**
NM_005952	MT1X	0.99 ± 0.05	1.02 ± 0.06	1.05 ± 0.06	**1.30 ± 0.09**	**1.40 ± 0.09**
NM_005953	MT2A	0.99 ± 0.05	1.02 ± 0.05	1.06 ± 0.05	**1.31 ± 0.08**	**1.40 ± 0.08**
NM_004510	SP110	1.03 ± 0.04	1.05 ± 0.04	1.10 ± 0.05	**1.17 ± 0.04**	**1.20 ± 0.03**
BC003388	TANK	1.02 ± 0.03	1.13 ± 0.04	**1.16 ± 0.04**	**1.29 ± 0.04**	**1.34 ± 0.06**
AB007447	TRAFD1	1.00 ± 0.02	1.04 ± 0.03	**1.10 ± 0.03**	**1.24 ± 0.03**	**1.28 ± 0.02**
AI831561	ZNRF2	0.99 ± 0.05	1.05 ± 0.05	**1.20 ± 0.04**	**1.26 ± 0.05**	**1.28 ± 0.06**
AK024296	ZBTB10	1.00 ± 0.04	1.09 ± 0.05	**1.16 ± 0.05**	**1.27 ± 0.05**	**1.29 ± 0.05**
NM_020119	ZC3HAV1	0.99 ± 0.05	1.10 ± 0.06	**1.14 ± 0.06**	**1.25 ± 0.06**	**1.31 ± 0.04**
NM_017742	ZCCHC2	1.00 ± 0.04	1.06 ± 0.05	**1.14 ± 0.05**	**1.14 ± 0.04**	**1.22 ± 0.04**
AA150460	ZNFX1	1.00 ± 0.02	1.05 ± 0.03	**1.11 ± 0.04**	**1.19 ± 0.03**	**1.23 ± 0.02**

*Fold change represents $\log_2$ mean intensities of infected /uninfected PBMC transcripts (two-way ANOVA, twofold cutoff; 5% FDR; $P \leq 0.05$ Tukey's HSD test) ($n = 8$).

†SEM = $\log_2$ SE of mean.

Genes/values in bold represent fold changes, which were statistically significant in this study.

Genes identified with gene ontology/*z*-score values (Materials and methods) (GeneSifter; VizXlabs.)

KP, Kegg pathway; BP, biological process; MF, molecular function.

Table 4. Identification of microarray genes expressed *in vivo*

	Fold change*							
	Time							
	This study (h)					*In vivo* tularemia (days)		
Gene ID	1 ± SEM†	4 ± SEM	8 ± SEM	16 ± SEM	24 ± SEM	2–3	6–7	13
ATP5C1	1.00 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	**0.98 ± 0.01**	0.99 ± 0.01	1.11	1.33	1.18
BAG1	1.01 ± 0.02	1.01 ± 0.01	**1.08 ± 0.03**	1.09 ± 0.02	1.05 ± 0.02	**−1.10**	**−1.47**	−0.47
BLVRB	1.01 ± 0.04	0.96 ± 0.04	0.91 ± 0.03	**0.84 ± 0.02**	**0.83 ± 0.02**	−0.38	**−1.29**	0.37
CASP1	1.00 ± 0.03	**1.04 ± 0.04**	**1.08 ± 0.04**	**1.15 ± 0.03**	**1.19 ± 0.03**	**1.10**	**0.70**	0.39
CCR7	1.01 ± 0.01	1.02 ± 0.02	1.03 ± 0.02	1.04 ± 0.02	1.06 ± 0.02	**−1.24**	−0.66	**−0.83**
CD3E	1.02 ± 0.02	1.00 ± 0.03	0.99 ± 0.03	0.99 ± 0.02	0.99 ± 0.02	**−1.12**	**−0.38**	0.13
CD8A	1.00 ± 0.04	0.99 ± 0.04	0.99 ± 0.04	1.01 ± 0.04	1.05 ± 0.03	**−1.00**	−0.45	−0.39
CD79A	1.01 ± 0.04	0.98 ± 0.03	0.978 ± 0.04	0.99 ± 0.04	1.01 ± 0.04	−1.05	**−1.22**	**−0.68**
COX7A2	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.01	1.01 ± 0.01	1.26	1.53	1.56
GIP2	1.01 ± 0.07	1.07 ± 0.08	1.17 ± 0.08	**1.26 ± 0.08**	**1.39 ± 0.06**	1.78	0.90	0.88
GBP1	1.03 ± 0.06	1.26 ± 0.13	**1.43 ± 0.12**	**2.12 ± 0.12**	**2.13 ± 0.08**	3.10	1.47	0.77
GCH1	0.99 ± 0.05	1.12 ± 0.07	**1.21 ± 0.06**	**1.38 ± 0.06**	**1.47 ± 0.05**	**1.90**	0.99	0.71
GLRX	1.00 ± 0.01	1.01 ± 0.02	1.02 ± 0.01	1.03 ± 0.01	**1.05 ± 0.02**	1.86	1.32	0.82
GNLY	1.01 ± 0.04	1.00 ± 0.04	1.01 ± 0.04	1.02 ± 0.04	1.07 ± 0.05	**−1.11**	0.34	**1.04**
GZMB	1.00 ± 0.04	0.99 ± 0.04	1.00 ± 0.04	1.13 ± 0.06	**1.34 ± 0.08**	−0.14	**1.19**	**1.47**
IFI16	1.01 ± 0.03	1.03 ± 0.03	1.06 ± 0.03	**1.08 ± 0.03**	**1.11 ± 0.02**	**1.83**	0.82	0.78
IFIT3	1.00 ± 0.08	1.14 ± 0.10	1.23 ± 0.09	**1.35 ± 0.09**	**1.51 ± 0.07**	**1.71**	0.55	10.14
IL2RB	1.00 ± 0.01	0.98 ± 0.02	1.00 ± 0.01	1.02 ± 0.01	1.09 ± 0.02	**−1.06**	**−0.64**	−0.27
LY96	1.01 ± 0.03	0.97 ± 0.03	0.94 ± 0.02	**0.93 ± 0.01**	0.94 ± 0.02	2.17	1.27	1.18
MSRB2	0.98 ± 0.04	0.97 ± 0.03	0.94 ± 0.03	1.01 ± 0.02	1.04 ± 0.03	**1.29**	0.44	0.30
PLAUR	1.00 ± 0.04	1.05 ± 0.05	1.10 ± 0.05	**1.21 ± 0.07**	1.16 ± 0.08	**1.43**	0.80	−0.22
PSME2	1.01 ± 0.04	1.00 ± 0.04	1.03 ± 0.03	**1.15 ± 0.02**	**1.21 ± 0.02**	1.62	1.04	0.74
RPL17	1.00 ± 0.01	1.00 ± 0.00	0.99 ± 0.01	0.97 ± 0.01	0.97 ± 0.01	0.63	1.04	1.18
S100A8	0.98 ± 0.05	1.04 ± 0.11	1.05 ± 0.06	1.08 ± 0.06	1.04 ± 0.09	**1.50**	**1.10**	0.53
SECTM1	1.01 ± 0.07	0.99 ± 0.06	1.00 ± 0.05	**1.17 ± 0.03**	**1.24 ± 0.04**	**1.73**	0.35	−0.24
SGK	1.00 ± 0.03	0.95 ± 0.04	0.94 ± 0.02	0.85 ± 0.03	**0.84 ± 0.02**	−1.31	−1.01	−0.67
SOD1	1.00 ± 0.01	0.99 ± 0.01	1.00 ± 0.01	0.99 ± 0.02	1.01 ± 0.02	0.49	0.75	**1.06**
SOD2	1.01 ± 0.07	**1.06 ± 0.06**	**1.14 ± 0.07**	**1.21 ± 0.05**	**1.09 ± 0.07**	**1.23**	0.58	0.39
STAT1	1.00 ± 0.05	1.02 ± 0.05	1.04 ± 0.04	**1.15 ± 0.02**	**1.20 ± 0.02**	**2.39**	1.31	0.54
TAP1	1.01 ± 0.04	1.04 ± 0.05	1.11 ± 0.05	**1.30 ± 0.03**	**1.35 ± 0.02**	**1.50**	**0.92**	0.09
TNFAIP6	0.94 ± 0.12	**1.38 ± 0.16**	**1.57 ± 0.16**	**2.14 ± 0.23**	**2.42 ± 0.27**	2.26	1.28	0.63
TNFSF10	1.01 ± 0.08	1.08 ± 0.11	1.18 ± 0.10	**1.32 ± 0.08**	**1.44 ± 0.06**	**2.22**	0.99	0.48
TOSO	1.03 ± 0.02	1.00 ± 0.02	0.97 ± 0.02	**0.93 ± 0.02**	0.94 ± 0.03	**−1.12**	−0.56	−0.33
TRGV9	1.01 ± 0.03	1.01 ± 0.04	1.03 ± 0.04	1.02 ± 0.03	1.03 ± 0.04	−1.03	0.74	1.68

*Fold change represents; $\log_2$ mean intensities of infected/uninfected PBMC transcripts two-way ANOVA, twofold cutoff; 5% FDR; $P \leq 0.05$ Tukey's HSD test) ($n = 8$).

†SEM = $\log_2$ SE of mean.

Genes/values in bold represent fold changes, which were statistically significant in this study.

In vivo significant gene expression values are indicated in bold (data from Andersson *et al*., 2006).

saw the mitochondrial gene Mn superoxide dismutase, SOD2, upregulated starting at 4 h as reported *in vivo* (Andersson *et al*., 2006). By contrast, Cu Zn superoxide dismutase, SOD1, had a very slight increase in expression by 24 h *in vitro*, though not significant in our study. GLRX, which plays a role in host defense in macrophages, was detected *in vivo* (Andersson *et al*., 2006) and induced *in vitro*. We detected significant upregulation of ATP5C1 at 16 h post infection. However, *in vivo* it was upregulated at all time points. The calcium-binding protein S100A8 was upregulated *in vivo*, and even though microarray gene expression indicated a slight induction, it was not significant according to our criteria.

SGK and TOSO were reported to be consistently downregulated in tularemia patients. *In vitro* infection resulted in significant downregulation of SGK and an overall trend in downregulation of TOSO (significant at 16 h) (Table 4). BLVRB was downregulated *in vivo* and *in vitro* (Table 4). In

contrast, some transcripts revealed opposite trends. BAG-1 was one such transcript that was downregulated in patients, but upregulated *in vitro*. In addition, four lymphocyte genes downregulated in tularemia (CD3 epsilon chain, CD79A, CCR7 and TRGV9) (Andersson *et al.*, 2006) did not change *in vitro*. Several genes with a pattern of early downregulation *in vivo*, such as T-cell effector granulolysin (GNLY), IL2RB, and CD8A, showed a tendency for increased transcription (although not statistically significant) in our study (Table 4). The opposite was true for T lymphocyte antigen gene MD-2 (LY96), which was downregulated *in vitro*, contrary to induction in tularemia patients (Andersson *et al.*, 2006). LY96 is required for lipopolysaccharide binding for signaling through TLR4, and the expression of TLR4 remained unchanged in our study (data not shown). A ribosomal gene RPL17 was reported to be upregulated in patients, even though in our study this gene remained unchanged.

Andersson *et al.* (2006) identified 13 potential biomarker genes uniquely regulated early in tularemia patients compared with convalescent patient samples and six of these genes belonged to the alarm signal. We identified five genes in this alarm signal cluster, namely caspase 1, PSME2, TAP-1, GBP1, and GCH1 (Table 4). Out of the seven biomarkers identified for tularemia by Andersson *et al.* (2006), we identified four being expressed in a temporal manner. TNFAIP6 was induced at 4 h and STAT1, TNFSF10, and SECTM1 at 16 and 24 h. We also detected MSRB2 with a slight increase in expression at 24 h but did not select it as significant according to our specifications. IL2RB and CD3E remained unchanged in our study (Table 4).

Novel genes/pathways identified during infection *in vitro*

We identified several genes in human PBMCs that had not yet been reported for *Francisella* infection (Table 3). Interestingly, two IFNα-induced genes that are part of the tryptophan metabolism pathway, namely indoleamine-2,3-dioxygenase (INDO) and tryptophan tRNA synthetase (WARS), were induced starting as early as 4 h post infection, together with KYNU and IBRDC2, which are associated with tryptophan metabolism.

Genes associated with lipid metabolism and the peroxisome proliferator-activated receptor (PPAR)-signaling pathway were also upregulated (Table 3). By 24 h post infection, several genes related to Ca^{2+} binding were both induced and downregulated. A significant number of genes associated with zinc binding, including several metallothionein genes, were upregulated during infection.

Discussion

We conducted microarray gene expression profiling of human PBMCs in response to *Francisella* infection and assessed to what extent host genes that have been reported in tularemia patients were also differentially expressed in our *in vitro* system. A very low initial infection rate of 1 : 10 bacteria : cells was used to study the early systemic phase of infection. At this MOI, we did not detect any genes that were differentially expressed at 1 h post infection, suggesting a threshold of infection for activation of host responses. Gene ontology analysis of microarray data revealed a number of cellular activities affecting host PBMCs by *Francisella* infection. Some categories of induced genes included binding, immune response, and apoptosis. We also found that a small number of genes in these categories were downregulated (cluster 2, Fig. 2). Interestingly, a group of genes was not affected by *Francisella* infection, although the genes' expression increased in uninfected PBMCs (cluster 4, Fig. 2). The reason for the stability of these transcripts as compared with uninfected controls is unknown and could be induced by either the host or bacteria. However, the stability may indicate such a requirement during infection.

The most prominent biological processes in tularemia were also found to be represented in our work, such as nucleic acid binding (77 genes), transcription factor activity (53), receptor activity (75), and hydrolase activity (59). IFNγ-responsive genes and a strong proapoptotic response (Table 2) were also observed both *in vitro* and *in vivo* (Andersson *et al.*, 2006).

The functional activities of differentially expressed genes (Fig. 1) were found to correlate well with those identified in the tularemia study by Andersson *et al.* (2006). These included hydrolase, oxidoreductase, and transcription factor activities and nucleic acid binding (Fig. 1). As reported by Andersson *et al.* (2006), IFNα-regulated genes were also upregulated in our study including GIP2, GBP1, GCH1, IFIT3, and IFI16 (Table 3). Also in agreement with published *in vivo* data (Andersson *et al.*, 2006), we saw the induction of a strong apoptotic response to *Francisella* infection (cluster 1, Table 2). In contrast, we observed early upregulation, and not downregulation, of immune response genes (Table 2). We detected significant induction of IL18, IL12p40, and IFNα genes within 24 h of infection (Table 2).

A few reports of *in vivo* studies on transcriptional analysis using microarrays for infectious diseases in humans are available (Kaushik *et al.*, 2005; Shao *et al.*, 2005). Our findings *in vitro* were in strong agreement with the human study of naturally occurring tularemia (Andersson *et al.*, 2006). For example, analysis of microarray data in both studies revealed similar ontology group categories and a considerable number of genes with similar expression patterns or trends. Some differences noted in terms of gene expression between the two studies may be explained partly by the fact that our study simulates the initial 'systemic' phase of infection with very early time points and the *in vivo* study focused on a more specific type of the disease, namely ulceroglandular tularemia, at a later time (Andersson *et al.*, 2006).

Innate immunity is a critical early response to *Francisella* infection that has been documented (Elkins *et al.*, 2003). Although we observed a strong early induction of immune responses to innate, acquired, or humoral immunity in our study, Andersson *et al.* (2006) found genes involved in these immune responses to be downregulated. One possible explanation for this discrepancy may be that, in tularemia patients, induction of these immune response genes may constitute an earlier event preceding the time when the samples were collected for the tularemia study.

Additionally, a comparison of the results of our *in vitro* study and previous findings showed that a number of other immune-related genes were differently affected. For example, CD3 epsilon chain and TRGV9 are T-cell-related genes that showed no changes in our study but were downregulated in the tularemia patients. CCR7, IL2RB, and CD8A, which showed increased expression (although not significant), are also T-cell-associated genes that were downregulated *in vivo*. A reasonable explanation could be found in the fact that in patients with ulceroglandular tularemia, the transient bacteremic phase may be over and thus immune responses more localized to lymph nodes, as reflected in circulating peripheral blood. Alternatively, host responses that occurred in both *in vitro* and *in vivo* studies were not synchronized in time.

Although we acknowledge the fact that these two studies (*in vitro* and *in vivo*) differed with respect to time points and the structure of controls, we successfully identified four out of seven potential biomarkers reported in the human tularemia study (Andersson *et al.*, 2006).

We used a whole human genome chip and were able to detect new pathways and cellular processes related to *Francisella* infection in the host. For example, we identified several genes associated with tryptophan metabolism/catabolism as early as 4 h post infection, which could be an indication of tryptophan regulation by the host following infection. Antigen-presenting cells regulate T-cell activation via tryptophan catabolism and the expression of INDO by these cells *in vivo* allows them to suppress unwanted T-cell responses (Munn *et al.*, 1999).

Genes associated with a role in lipid metabolism and PPAR pathways were also found to be mostly induced at a later time point of 16 h post infection. More specifically, APOL1 through 4 and APOL6 were upregulated during infection (Table 3). It has been found that APOL1 kills African trypanosomes, and APOL expression has been documented in various pathological processes (Vanhollebeke & Pays, 2006). Lipid metabolism may play a role in *Francisella* infection, as rabbits infected with *F. tularensis* show drastic changes in serum lipid content (Farshtchi & Lewis, 1968).

Ca^{2+}, one of the many universal-signaling mediators in cells which facilitate cross-talk between pathways, helps to integrate and fine-tune the overall innate response. Changes in intracellular Ca^{2+} homeostasis can lead to cell death, either through apoptosis or necrosis (Berridge *et al.*, 2000). Infections with intracellular pathogens, such as *Toxoplasma gondii* (Masek *et al.*, 2006), *Salmonella* (Gewirtz *et al.*, 2000), and mycobacteria (Yadav *et al.*, 2004), have identified a role for Ca^{2+} in facilitating proinflammatory signaling. In our study, the finding of differentially expressed genes associated with calcium ion binding may indicate a role for host calcium ion signaling relating to *Francisella* infection.

We also identified many genes associated with zinc ion-binding activities with several functional isoforms of metallothionein transcripts (MT-1 and MT-2) upregulated starting at 16 h post infection (Table 3). While the mechanisms of zinc ion interaction with immune cells are still poorly understood, zinc homeostasis is associated with immunoregulation (Rink & Haase, 2007). It was very interesting that during early *F. tularensis* infection in rats, lower levels of plasma zinc were detected and provided evidence that metallothioneins are intimately involved in the zinc redistribution occurring during the acute stage of illness (Sobocinski *et al.*, 1978).

The present study has identified novel host pathways potentially associated with *Francisella* infection. The relevance of our findings for *F. tularensis* infection needs to be further evaluated in future studies as it is beyond the scope of the present study which investigated the aspects of global transcriptional changes in infected PBMCs. A major goal in our study was to test the assumption that microarray technology could produce gene expression data, which reflects the infectious process in tularemia patients. Even though the time points of our observations were not similar to those in tularemia patients, results from our work indicate that gene expression studies performed *in vitro* could provide valuable information regarding the infectious processes occurring *in vivo*. Additionally, our study demonstrates that transcriptional profiling *in vitro* could be used as a reliable tool to identify potential biomarkers which conform to human tularemia. This work relied on the use of a comprehensive array assessing gene expression changes in the entire human genome. This capability was critical in helping us identify several new host pathways that may potentially be implicated in the early phase of *Francisella* infection, and, therefore, merit further investigation.

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